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Signal amplification of padlock probes by rolling circle replication

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ABSTRACT

Circularizing oligonucleotide probes (padlock probes) have the potential to detect sets of gene sequences with high specificity and excellent selectivity for sequence variants, but sensitivity of detection has been limiting. By using a rolling circle replication (RCR) mechanism, circularized but not unreacted probes can yield a powerful signal amplification. We demonstrate here that in order for the reaction to proceed efficiently, the probes must be released from the topological link that forms with target molecules upon hybridization and ligation. If the target strand has a nearby free 3′ end, then the probe–target hybrids can be displaced by the polymerase used for replication. The displaced probe can then slip off the target strand and a rolling circle amplification is initiated. Alternatively, the target sequence itself can prime an RCR after its non-base paired 3′ end has been removed by exonucleolytic activity. We found the Φ29 DNA polymerase to be superior to the Klenow fragment in displacing the target DNA strand, and it maintained the polymerization reaction for at least 12 h, yielding an extension product that represents several thousand-fold the length of the padlock probe.

INTRODUCTION

Molecular genetic research is in transit from a state where selected regions of genomes could be cloned and used as hybridization probes, to one where digital information increasingly becomes available for whole genomes, including in just a few years that of humans. In order to take advantage of this development, new probing techniques will be required to simultaneously analyse very large sets of gene sequences with sufficient selectivity to resolve single nucleotide differences. Short oligonucleotide hybridization probes of ~20 nt can distinguish single nucleotide differences (1), but they lack specificity to detect unique sequences in complex genomes. By contrast, the polymerase chain reaction (PCR) achieves adequate specificity by requiring co-ordinated hybridization by pairs of oligonucleotide probes, but the diffusible reaction products and problems of simultaneously analysing large sets of sequences represent important limitations.

Circularizing oligonucleotide probes (padlock probes) are promising reagents for advanced genetic analyses. The 5′ and 3′ end regions of these linear oligonucleotides are designed to base pair next to each other on a target strand. If properly hybridized, then the ends can be joined by enzymatic ligation, converting the probes to circularly closed molecules that are catenated to the target sequences (2). These probes are suitable for simultaneous analysis of many gene sequences due to their manifest specificity and selectivity for gene sequence variants (3,4), and because the reaction products remain localized at the target sequences. Moreover, intramolecular ligation of many different probes is expected to be less susceptible to crossreactions than multiplex PCR where non-cognate pairs of primers can give rise to irrelevant amplification products (5).

What has been lacking in gene detection using padlock probes is a means to amplify detection signals from reacted probes. Several groups have demonstrated that circular oligonucleotides can support a rolling circle replication (RCR) reaction, analogous to replication mechanisms of several viruses with circular genomes (6,7), and the method has been applied for amplified detection of viral RNA from tissue samples (8) and for preparative *in vitro* synthesis of catalytic antisense RNA (9). Through the RCR reaction, a strand can be generated that represents many tandem copies of the complement to the circularized molecule. Lizardi *et al*. (4) have recently used RCR to obtain an isothermal cascade amplification reaction of circularized padlock probes *in vitro* in order to detect single-copy genes in human genomic DNA samples. They also detected single DNA molecules in a solid phase based assay but report difficulties in doing the same in *in situ* hybridization reactions. We show here that the topological link formed between a padlock probe and its target sequence inhibits RCR, and that this inhibition can be circumvented if a free end is introduced in the target DNA strand close to the probe ligation site. Because of its high strand displacement activity (10), the DNA polymerase from the *Bacillus subtilis* phage Φ29 can release probes from a target sequence by allowing them to slip off a nearby end. Thereby a powerful signal amplification of individual probe molecules can be achieved.

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

MATERIALS AND METHODS

Oligonucleotides

A 70mer oligonucleotide probe (5′-TGCCTGCAGGTCGACTTT-TTTTTTTATGTTAAGTGACCGGCAGCATTTTTTTTTTAGT-GCCAAGCTTGCA-3′) was synthesized using standard phosphoramidite chemistry on an ABI 394 DNA Synthesizer. The probe was chemically 5′-phosphorylated (11) and purified by reversed-phase chromatography. The 15 nt at each end of the probe were designed to hybridize to two immediately adjacent segments in the polylinker region of the M13mp18 single-stranded cloning vector (M13) (positions 6263–6292). A primer P1 (5′-TGCTGCC-GGTCACTTAACAT-3′), and two oligonucleotide templates for ligation, T30 and T50, identical to the vector sequences 6263–6292 and 6258–6297, respectively, with the latter further extended by five T-residues at both the 5′ and 3′ ends, were synthesized as above but without 5′ phosphorylation. Two more oligonucleotides; O1, complementary to P1, and O2, complementary to M13 sequence 2640–2659, were purchased from Gibco BRL. O2 was tailed using terminal deoxynucleotide transferase (Amersham), and an 8-fold molar excess of dideoxyNTP (Pharmacia Biotech), and purified through a Sephadex G-50 spin column (Pharmacia Biotech). M13 DNA was from Amersham.

Conditions for radiolabeling, ligation, polymerization and restriction digestion

Aliquots of 10 pmol of probes or primers were 5′-radiolabeled using 30 U of T4 polynucleotide kinase (Amersham) in 50 µl 50 mM KAc, 10 mM $MgAc_2$, 10 mM Tris–Ac (pH 7.5) and 50 µCi [γ-^{32}P]ATP (3000 mCi/mmol, NEN DuPont) at 37°C for 30 min. The enzyme was heat-inactivated at 65°C for 10 min, and oligonucleotides were purified on a Sephadex G-50 spin column.

Padlock probe ligation reactions contained a probe–target ratio of 1:3 (4 nM probe in the experiment reported in Fig. 3, 10 nM in Figs 1 and 4, and 50 nM in all others), 50 mM KAc, 10 mM $MgAc_2$, 10 mM Tris–Ac (pH 7.5), 1 mM ATP, 0.1 µg/µl bovine serum albumin (BSA) and 0.5 U/µl T4 DNA ligase (Amersham) in a volume of 20 µl. The reactions were heated to 65°C and cooled to room temperature before addition of ligase, followed by incubation at 37°C for 1 h. Ligation was terminated by incubation at 65°C for 10 min.

Aliquots of 4 µl of ligation reactions were used to template RCR reactions in a final volume of 20 µl of 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 20 mM $(NH_4)_2SO_4$, 1 mM dithiothreitol and 0.2 µg/µl BSA, 0.25 mM dNTP, 5 µCi [α-^{32}P]dCTP (3000 Ci/mmol, NEN DuPont) and 2 ng/µl Φ29 DNA polymerase (a kind gift from Dr John Cunniff, Amersham, Cleveland, OH). Reactions catalyzed by the Klenow fragment of DNA polymerase I from *Escherichia coli* (Amersham) were performed in 20 µl 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, 1.4 µg BSA and 2.5 U of the polymerase. All RCR reactions were primed using 0.1 pmol unlabeled or 30 fmol radiolabeled oligonucleotide P1, except those where the T30 oligonucleotide was used both to template ligation and to prime polymerization. The reactions were heated to 65°C and cooled to room temperature prior to addition of polymerase, followed by incubation at 37°C for the indicated times. Reactions were terminated by incubation at 65°C for 10 min.

Single-stranded M13 DNA was linearized by rendering the *Xmn*I site (positions 2640–2659) double-stranded using a 4-fold molar excess of the dideoxy-terminated oligonucleotide O2 and incubating for 22 h at 37°C in the presence of 12 U of the restriction enzyme *Xmn*I (New England Biolabs).

RCR products were restriction digested by adding the complementary oligonucleotide O1 to the polymerization reactions, and adjusting the buffer to conditions recommended by the manufacturer. Reactions were heated to 65°C and cooled to room temperature prior to the addition of 10 U of the restriction enzyme *Hpa*II (New England Biolabs). Digestions were performed at 37°C overnight, and stopped by incubation for 10 min at 65°C in an equal volume of stop buffer (75% formamide, 10 mM EDTA, 5 mg/ml SDS, 0.5 mg/ml bromophenol blue and 0.5 mg/ml xylene cyanol). Samples were separated in denaturing

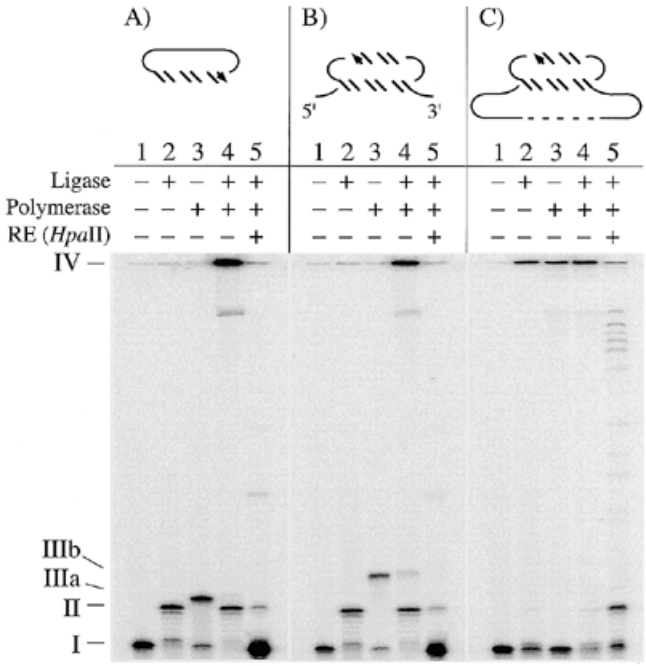

Figure 1. The efficiency of RCR of padlock probes depends on the presence and the nature of the target strand for probe ligation. (**A**) RCR primed by oligonucleotide T30, also used to template probe ligation. (**B**) RCR of a padlock probe, hybridized to target oligonucleotide T50 that is extended by 10 nt 5′ and 3′ of the probe-complementary region. (**C**) Attempted replication of a padlock probe, catenated to a circular single-stranded target molecule. Reaction products were separated in a denaturing 6% polyacrylamide gel. In all lanes 5′-radiolabeled padlock probes are seen, along with any RCR products labeled through incorporation of radioactive nucleotides. Lane 1 in each panel illustrates the unmodified padlock probe. Lane 2 demonstrates the results after addition of ligase and unlabeled target molecules. In lane 3 a polymerization reaction was performed without prior probe ligation. In lane 4 probes were subjected to ligation, followed by a 30 min polymerization reaction. Finally, lane 5 represents reactions where probes were ligated and allowed to template replication, after which the polymerization products were hybridized to an oligonucleotide and restriction digested to generate monomer fragments. The roman numeral I identifies bands corresponding to unligated padlock probes and digested rolling circle products. II corresponds to free circularized padlock probes. IIIa and IIIb are extension products from the unligated padlock probes, templated by the targets in (A) and (B), respectively. IV represents molecules too large to enter the gel and include padlock probes catenated to M13 target sequences, RCR products, and extension products from unligated padlock probes, templated by M13 DNA. Arrowheads in the models denote 3′ ends of primers for polymerization. RE, restriction enzyme.

polyacrylamide gels, and radiolabeled bands were quantified on a PhosphorImager (Molecular Dynamics).

Measurement of the rate of RCR

Oligonucleotide T30 was used to template probe circularization and to prime replication. Reaction components omitting dATP were assembled and brought to 37°C. The reaction was then initiated by the addition of dATP. The rate of RCR was obtained by comparing incorporation of radioactive nucleotides during a 1 min incubation in, on the one hand RCR products digested to monomer size, and on the other the extension products from unligated probe–target hybrids (corresponding to band I in lane 5, and band IIIa in lane 3, respectively, in Fig. 1A). The measurement was corrected for ligation efficiency and for the difference in nucleotide composition of the different products.

RESULTS

RCR of circularized padlock probes

RCR reactions are ideally suited to specifically amplify the detection signal from padlock probes that have reacted in a target-sequence-dependent manner. Concatenated complementary sequences can only be generated from circularized padlock probes. In contrast, any remaining unreacted probes only template synthesis of short base-paired sequences not available for hybridization detection. Probes circularized in a target-dependent ligation reaction were efficient templates for RCR when the target sequence was also used to prime DNA synthesis by the highly processive DNA polymerase from phage Φ29 (Fig. 1A). The polymerization products were too large to enter denaturing polyacrylamide gels, but their identity was confirmed by restriction digestion to fragments of a monomer size corresponding to that of the padlock probes.

We estimated a mean incorporation rate of 1.0×10^3 nt/min when the RCR reaction was primed by an oligonucleotide that also served as the ligation template, as in Figure 1A (data not shown). This Φ29 DNA polymerase-catalyzed RCR reaction proceeded for at least 12 h with an estimated half-life of 11 h (Fig. 2). This means that each circularized probe templated synthesis of a single-stranded product 14 times the length of the probe each minute, yielding several thousand-fold amplification of reacted padlock probes in an overnight incubation, corresponding to ~0.5 Mb of DNA synthesized per probe.

Inhibition of replication by different target molecules

In practical applications, padlock probes will be reacted with a target sequence before an RCR can be initiated. We found that the nature of the connection of a padlock probe to its target DNA strand greatly influences the efficiency of the reaction. Thus, no RCR product was obtained when the primer for the replication reaction hybridized to a circularized probe, which in turn was catenated to the circular single-stranded M13 cloning vector (Fig. 1C). The molecules that fail to enter the gel in Figure 1C represent padlock probes catenated to M13 target sequences (2) (lanes 2 and 4) and extension products from unligated padlock probes, templated by M13 DNA (lanes 3 and 4). When unlabeled probes were used, no monomer-sized fragments appeared after restriction digestion (data not shown and Fig. 3). By contrast, a prominent RCR product was obtained (Fig. 1B) when the target molecule extended beyond the probe-complementary region by 10 nt at

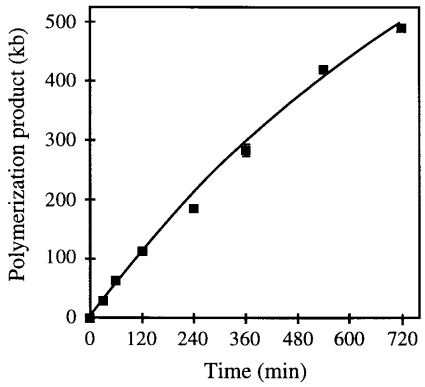

Figure 2. Kinetics of RCR product accumulation. Padlock probes were circularized by hybridizing to oligonucleotide T30 that also served as primers for the Φ29 DNA polymerase. The radiolabeled polymerization products were digested to monomer size and separated by gel electrophoresis and the mean band intensities of triplicate determinations were calculated. The results were converted to an estimated average length of DNA synthesized per circularized probe, assuming an initial incorporation rate of 1000 nt/min, and plotted against reaction time. The solid line describes an exponentially decaying polymerization rate having a half life of 11 h.

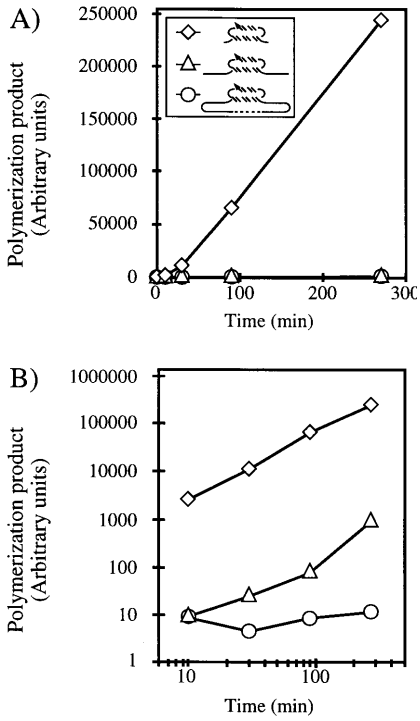

Figure 3. A comparison between the progress of RCRs templated by padlock probes hybridized to the following three different templates: oligonucleotide T50 extending by 10 nt 5′ and 3′ of the probe-complementary sequence (diamonds); an M13 single-stranded molecule linearized through restriction digestion to yield free ends located 3.6 kb 5′ and 3′ of the site of probe binding (triangles); and a circular M13 molecule (circles). Radiolabeled polymerization products were digested to unit size and separated by gel electrophoresis. The intensities of the relevant bands were plotted versus duration of the RCR reaction. The results are shown (**A**) as a lin–lin plot, and (**B**) as a log–log plot to allow comparison between all different reactions.

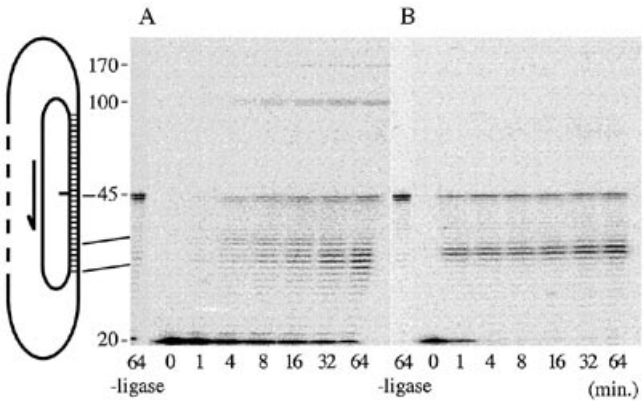

Figure 4. Displacement of the padlock probe–target hybrid by a 5′-radiolabeled primer using (**A**) Φ29 DNA polymerase and (**B**) the Klenow fragment of *E.coli* DNA polymerase I. Padlock probes were hybridized and circularized on circular M13 single-stranded vectors and elongation of labeled primers was monitored by separation in a denaturing 10% polyacrylamide gel after the indicated incubation times. Molecular sizes in nucleotides were estimated by comparison to *Pst*I-digested λ-DNA. Primer extension to the 5′-end of remaining non-ligated probes generated products of 45 nt.

both the 5′ and 3′ ends. The 3′→5′ exonucleolytic activity of the Φ29 DNA polymerase (12) can allow the target molecule to prime RCR by first removing 3′ non-probe-complementary nucleotides (data not shown). However, the same target sequence protected from exonucleolysis by including phosphorothioates at the five 3′-most phosphodiester bonds also supported RCR, suggesting a strand releasing mechanism (data not shown). This rules out 3′→5′ exonucleolysis as the sole mechanisms permitting replication of padlock probes hybridized to short target molecules (Discussion).

We further compared the accumulation of RCR products over time for padlock probes previously reacted with various target sequences. Figure 3 demonstrates a slight delay before rapid accumulation of RCR products started when padlock probes were hybridized to target sequences that extended by 10 nt from either end of the probe-complementary region. This is in contrast to the replication of a free circular probe as seen in Figure 2, where no initial delay was evident. When probes were ligated to M13 molecules linearized such that this target molecule extended by ~3.6 kb both 5′ and 3′ of the target-complementary region, then replication was greatly inhibited, but also under these conditions did the rate of replication increase over time. Finally, probes catenated to circular M13 DNA failed to template replication of measurable amounts of rolling circle products (Fig. 3). This situation, where padlock probes are hybridized to a target molecule with very distant or unavailable free ends, is probably representative of most conditions under which padlock probes might be applied.

Mechanism of inhibition of RCR

The progress of an attempted RCR of a probe linked to a circular target molecule, the M13 cloning vector, was examined in greater detail by labeling the 5′ end of the primer and observing the accumulation of extension products by gel separation. Figure 4 demonstrates that the Φ29 DNA polymerase, unlike the Klenow fragment of *E.coli* DNA polymerase I, can to some extent displace the base-paired target segment and replicate the circularized probe. Stops at 5–7 nt into the base-paired segment are seen with both polymerases, but relatively weak additional bands of sizes ~100 and 170 nt, increasing in intensity over time, are seen only when the Φ29 DNA polymerase was used. The size of these bands indicates that the polymerase has proceeded around the probe one or two laps before being brought to a halt. The results illustrate that the polymerase can replicate the padlock probe by displacing a long target sequence. Apparently, replication beyond one or two turns around the probe is inhibited, perhaps due to molecular crowding of the target strand that remains threaded in the circularized probe (Fig. 5A).

DISCUSSION

Padlock probes are highly specific reagents that should be suitable to distinguish single nucleotide sequence variants in complex genomes such as that of humans. We demonstrate herein that detection of the circularized probes can be augmented by an efficient RCR. In this manner, strands can be generated that include thousands of copies of the complement of the probe. Moreover, these replication products are synthesized at a uniform rate and in a single-stranded form that is directly available for detection and quantification via specific hybridization probes. Furthermore, the length of the RCR products should serve to limit diffusion during *in situ* detection reactions. However, any applications of the RCR mechanism must take into account that the reaction is greatly inhibited if the probes are bound to target sequence with no nearby free ends.

During RCR of short padlock probes only part of the probe can be base-paired at any given time. This is because double-stranded DNA is quite rigid and cannot form circles of <150 bp unless a bending force is applied (13). This implies that during RCR of short DNA circles it is the strain in the bent DNA helix, rather than the direct action by the polymerase, that causes the polymerization product to be continuously displaced. Accordingly, also polymerases with 5′→3′ exonuclease activity can produce long polymerization products when replicating short circles (6,7). The proportion of double-stranded DNA in the circular probe during RCR can be calculated from the maximal bending of double-stranded DNA (360°/150 bp) and the length per nucleotide in double-stranded (0.34 nm) and single-stranded DNA (0.59 nm) (14). For example, we estimate that out of the 70 nt in our probe, a length of 46 contiguous nucleotides are base-paired at any time during RCR. Because of its size, the polymerase is unlikely to progress through the DNA circle during replication, but rather it is expected to move along the periphery of the circle, rotating the double-stranded segments in a counterclockwise direction as it goes along. As a consequence, the displaced end of the RCR product is continuously released, preserving the number of times the product is threaded through the circularized padlock probe.

Efficient RCR is initiated as soon as a circular probe is released from its target strand. This occurs fast if the distance to the nearest end of the target strand is short, very much slower if it is measured in kb, and not at all if the ends of the target are unavailable such as when the target is also circularly closed.

The Φ29 DNA polymerase can dissociate the probe–target hybrid and replicate the circularized probe once or twice also when the probe remains linked to a circular target strand. The Klenow polymerase, by contrast, fails to displace linked probes from target DNA strands. This difference could be due to the fact

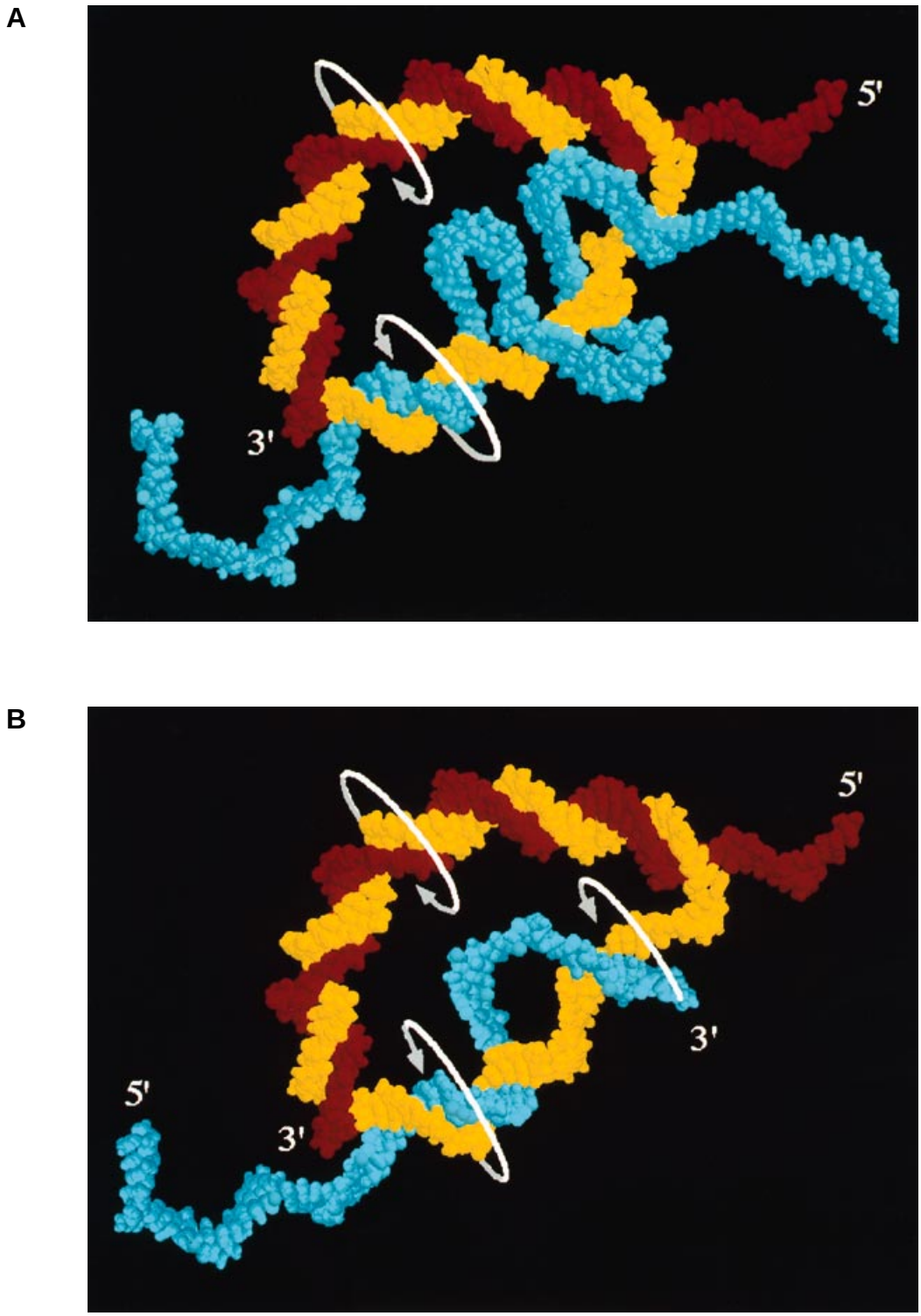

Figure 5. Molecular model of RCR of padlock probes hybridized to two different target molecules. The padlock probes are shown in yellow, the target strands are blue and the primers being extended are red. Polymerization takes place at the 3′ end of the primers that in both panels have been extended by ~30 nt, and displaced 20 nt of the probe–target duplex. The extension induces a rotation of the probe-target and probe-extension product helices (arrows). (**A**) Replication of a padlock probe bound to a long or circular target strand. The polymerase displaces the 5′ part of the target strand, but since it has no free end nearby, the target strand remains coiled around the probe. (**B**) Replication of a padlock probe that is base-paired to a short target strand, extending by 10 nt from both ends of the target-complementary segment. The polymerase displaces the target strand at its 5′ end, and the rotation induced by the polymerase causes the 3′ end of the target to unwind through the probe circle until it is released.

that the two polymerases differ in their processivity and their capacity for strand displacement. While the Klenow polymerase on average adds 7.7 nt before it dissociates from its template (15), the Φ29 DNA polymerase replicates the 19 kb double-stranded Φ29 genome in 8 min without dissociating from the template (10).

Once the Φ29 DNA polymerase has extended a primer beyond the target-complementary part of the padlock probe, then there is no specific hybridization to the target strand and the padlock probe is free to diffuse along the target strand. We have previously shown that catenated padlock probes can migrate considerable distances along target DNA strands under conditions where base pairing is prevented (2). A circular target strand cannot be released by this mechanism, leaving the target strand still threaded through the circularized probe and inhibiting RCR (Fig. 5A).

In contrast, replication of a circular probe that is hybridized to a target DNA strand with a nearby free end is almost as efficient as replication reactions where the target also acts as a primer. This could be because the 3′ ends of short target DNA strands are passed through the probe circles through the rotation of the probe–target helix, induced by the displacing activity of the progressing Φ29 DNA polymerase (16). Thereby, the number of times the target strand is threaded through the padlock probe decreases until the target is released (Fig. 5B). Alternatively, 3′ non-complementary nucleotides are removed by 3′→5′ exonucleolysis, thereby allowing the target strand to act as a primer for RCR. In the latter case it is notable that the RCR product becomes contiguous with the target sequence. This may be of value if a localized signal is desired.

In conclusion, RCR can provide a powerful, local signal amplification of specifically reacted padlock probes. This replication should be valuable for *in situ* detection of single-copy gene sequences and to enhance sensitivity in array-based analyses of multiple gene sequences. In order to take advantage of the potential for efficient signal amplification via RCR, the target strands must have a free end close to where the padlock probe hybridizes. It will be of importance to develop methods to fragment target DNA without destroying chromosome morphology or having the probe and its replication product diffuse away from the target sequence, thereby causing loss of positional information.

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